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Multiple forms of cytochrome *P*-450 related to forms induced marginally by phenobarbital

Differences in structure and in the metabolism of alkoxyresorufins

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The properties of five structurally related forms of cytochrome *P*-450 (PB_{1a}, PB_{1b}, PB_{2a}, PB_{2b} and PB_{2d}) isolated from rats treated with phenobarbital have been compared with two forms isolated previously now termed 'PB_{1c}' and 'PB_{2c}'. These enzymes were characterized by their marginal inducibility by phenobarbital and are clearly distinguishable from the major phenobarbital-inducible proteins. PB_{1a} and PB_{1b} differed in M_r (52700 and 52900), absorption spectra and papain-proteolysis fragments. However, they had identical *N*-terminal sequences. PB_{2a}, PB_{2b} and PB_{2d} had apparent M_r values of 52900, 52900 and 50800. PB_{2a} and PB_{2b} had different *N*-terminal sequences and, after digestion with papain, gave different papain-proteolysis fragments. The *N*-terminal sequence of PB_{2b} was similar to, but not identical with, that of pregnenolone-16α-carbonitrile-inducible *P*-450 species, and PB_{2b} was the protein most closely related to PB_{2c}. The extent of immunocross-reactivity among the forms was stronger within, than between, the PB_1 and PB_2 groups. Even structurally similar forms were functionally diverse, exhibiting large differences in metabolic specificity in the dealkylation of a series of alkoxyresorufins.

INTRODUCTION

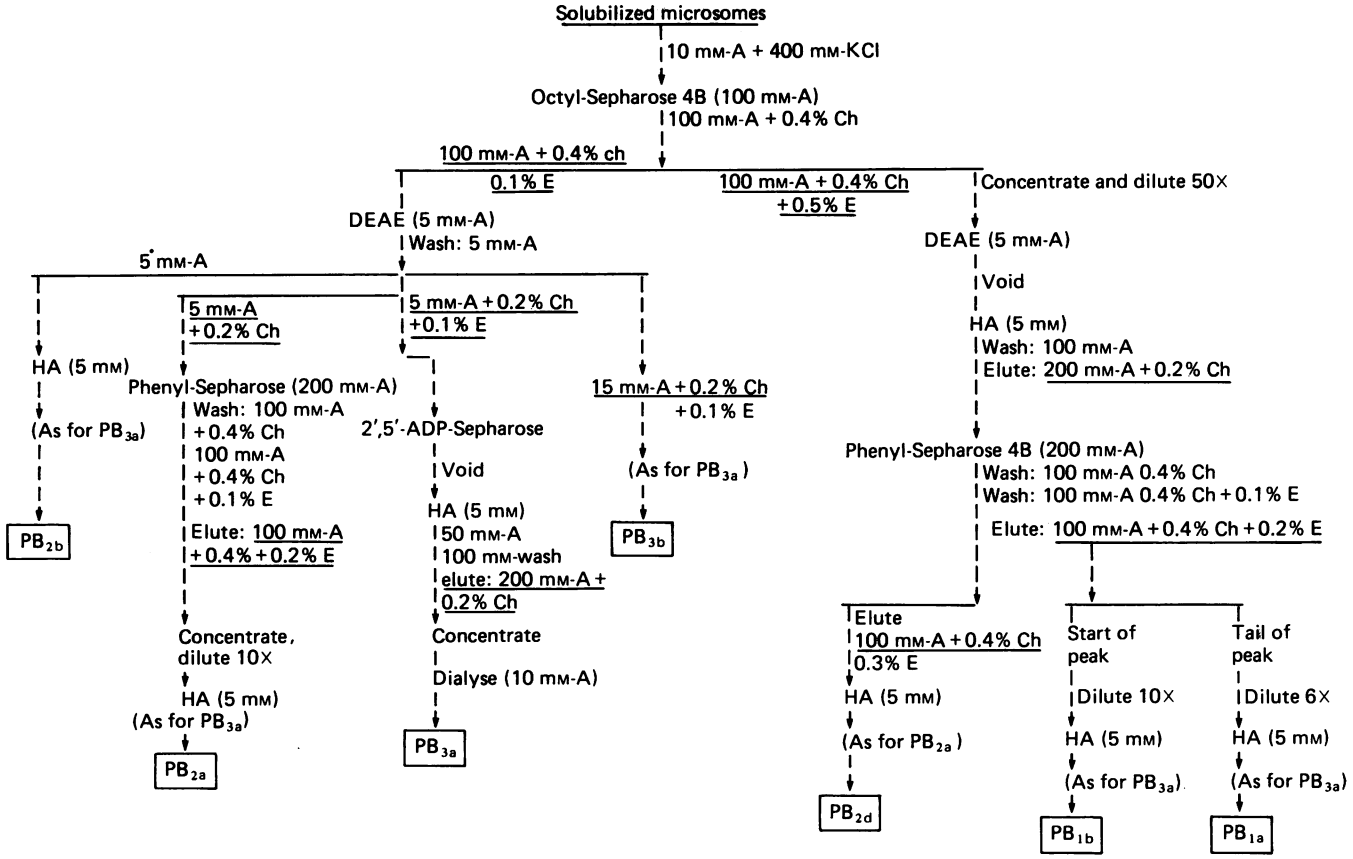
The cytochrome *P*-450 mono-oxygenases represent a complex supergene family of proteins that play a central role in the metabolism of drugs, carcinogens, steroid hormones and fatty acids [1,2]. This family has been split into several subfamilies [3,4]. Those involved in foreign-compound metabolism have been classified by their inducibility in the liver by specific compounds including 3-methylcholanthrene [1,3,4], high and low inducibility with phenobarbital [1,3,5] and by inducibility with pregnenolene-16α-carbonitrile [6,7]. Members within the same subfamily can show extensive sequence homology (up to 97% [8,9]), yet large differences in substrate specificity have been observed [10]. The recent cloning of cytochrome *P*-450 genes has shown that there may be a large number of genes or pseudogenes within a cytochrome *P*-450 subfamily [3,4]. This has raised the question of which genes are actually expressed and how substrate specificity of the gene products relates to drug metabolism, chemical toxicity and carcinogenicity. One of the gene subfamilies in which we are interested is constitutively expressed and characterized by marginal induction by phenobarbital. Members of this family have been isolated from rat liver and termed 'PB_1' by Waxman & Walsh [11] and 'PB-C' by Guengerich *et al.* [6]. We have previously described the isolation and purification of two cytochrome *P*-450 forms (PB_1‖ and PB_2) marginally inducible by phenobarbital and a variety of other reagents such as *trans*-stilbene oxide and Aroclor 1254 [12]. Interestingly, PB_1 also appears to be elevated at early time points in preneoplastic foci in rat liver [13]. Proteins structurally related to PB_1 and PB_2 are expressed in high levels in human liver, but with significant inter-individual variation [14]. In order to identify structurally related forms of PB_1 and PB_2 we have isolated several cytochrome *P*-450 forms from rat liver and have compared their properties.

MATERIALS AND METHODS

Methods

Cytochrome *P*-450 forms were isolated from hepatic microsomal fractions of male Wistar rats (200 g) treated on 3 consecutive days before use with sodium phenobarbital (80 mg/kg, intraperitoneally). Microsomal samples were diluted to a protein concentration of 10 mg/ml with 10 mM-phosphate buffer, pH 7.7, and solubilized by the addition of sodium cholate [1 mg/mg of protein; stock solution 10% (w/v) in water] for 30 min at 4 °C. Glycerol was added to the solubilized sample [final concn. 20% (v/v)] and the ionic strength of the mixture increased to 400 mmol/litre by the addition of 1 M-KCl. This sample was then applied to a column (5 cm × 100 cm) of octyl-Sepharose 4B (approx. 10 nmol of cytochrome *P*-450/ml of column volume) equilibrated with 200 mM-phosphate buffer, pH 7.7, containing 20% (v/v) glycerol, 0.1 mM-dithiothreitol and 0.1 mM-EDTA (Buffer A). The column was washed with this buffer containing 0.4% cholate, then 100 mM-Buffer A contain-

‖ On the basis of the information then available, originally PB_1 was thought to be equivalent to form b described by Ryan *et al.* [10,12,13]. However, *N*-terminal sequence analysis shows this protein to be structurally related to that described by Waxman & Walsh [11] as PB_1.



Scheme 1. Flow diagram for the method used in the purification of the Cytochromes *P*-450

Liver microsomes of phenobarbital-treated rats were used. Abbreviations used: A, Buffer A; HA, hydroxyapatite; E, Emulgen 911; Ch, sodium cholate. Equilibration buffers are given in parentheses. 'Underlined' solution mixtures were those used for the elution of cytochrome fractions. 'As for PB_{3a}' means that the purification procedure then followed that described for the preparation of PB_{3a} from the final hydroxyapatite column onwards. DEAE, hydroxyapatite and octyl- and phenyl-Sepharose columns were loaded at 20, 30, 10 and 10 nmol of cytochrome *P*-450/ml of column material respectively.

ing 0.4% cholate. Cytochrome fractions were then eluted, first with 100 mM-buffer A containing 0.4% cholate and 0.1% Emulgen 911. Cytochrome *P*-450 was eluted as a broad peak and was split into three pools. The initial fractions contained form PB_{2a}, the major middle fraction contained forms PB_{3a} and PB_{3b} (equivalent to forms b and e [5]) and the tail contained form PB_{2b} (Fig. 1 below). Buffer A containing 0.4% cholate plus 0.5% Emulgen 911 was then applied to the column and further cytochrome fractions were eluted containing forms PB_{1a}, PB_{1b} and PB_{2d}. At this, and all subsequent, stages of the purification only the purest cytochrome fractions of equivalent mobility, assessed by SDS/polyacrylamide-gel electrophoresis, were pooled. Other details of this procedure are given in Scheme 1. The specific content of the cytochrome samples obtained were 22.7 (PB_{1a}), 16.0 (PB_{1b}), 15.7 (PB_{2a}), 7.4 (PB_{2b}), 13.9 (PB_{2d}), 15.2 (PH_{3a}) and 16.6 (PB_{3B}) nmol/mg of protein. The 418 nm/280 nm absorption ratio of the proteins was greater than 3:2, with the exception of PB_{2b} and PB_{2d}. These samples did contain minor impurities, and in the case of PB_{2b} there appeared to be some loss of haem during purification. PB_{1c} and PB_{2c} were purified as described previously [12] and had specific contents of 16.4 and 15.2 nmol/mg of protein. Protein determinations were carried out by the method of Lowry *et al.* [15], with bovine serum albumin as standard. SDS/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli [16]. The proteolytic digestion of the proteins with papain, followed by SDS/polyacrylamide-gel electrophoresis, was by the method of Cleveland *et al.* [17] as described by Slaughter *et al.* [18]. Samples (75 μg of protein; 150 μl) were incubated with papain (25 μl of stock; 1 mg/ml) and aliquots (30 μl) were removed at 0.5, 2.0, 5.0, 20 and 60 min. The reaction was stopped by the addition of 24 μl of quenching reagent [7.5% (v/v) mercaptoethanol/2.5% (w/v) SDS] and the sample boiled for 2 min. The entire sample (11.2 μg) was taken for electrophoresis. The reconstitution of monooxygenase activity was carried out as described previously [19], with the exception that sodium cholate was omitted and the preincubation time was 5 min. Benzphetamine *N*-demethylation was measured by the rate of formaldehyde formation as described by Nash [20]. Incubations were for 15 min at 37 °C. Purified cytochrome *P*-450 (0.1 nmol), 1000 units of *P*-450 reductase and 50 μg of didodecyl phosphatidylcholine were taken for each incubation. Cytochrome *P*-450 reductase was prepared as described previously [21]. The *O*-de-ethylation of 7-ethoxycoumarin was measured dynamically by the method of Ullrich & Weber [22]. The *O*-dealkylation of alkylphenoxazones (alkoxyresorufins) and the hydroxyl-

ation of phenoxazone was measured dynamically at 37 °C, resorufin formation being monitored as described by Burke & Mayer [23].

N-Terminal sequencing was carried out by Mr. Bryan Dunbar at the sequencing facility in the Department of Biochemistry, University of Aberdeen, Aberdeen, Scotland, U.K., in a Beckman 890C liquid-phase sequencer fitted with the Beckman cold-trap accessory, by using the 0.25 m Quadrol program with Polybrene. Some samples (PB_{2a} and PB_{2b}), available in only small quantity, were sequenced in the Applied Biosystems 470A gas-phase machine using the 02NVAC or 02cPTH program with an argon atmosphere. Identification of phenylthiohydantoin derivatives was by reverse-phase h.p.l.c. on an Apex cyano column (0.45 cm × 25 cm) with an acetate/acetonitrile, pH 5.0, system.

Antibodies to the cytochrome *P*-450 isoenzymes were raised in rabbits as described previously [19]. Dot blots were carried out by spotting 1 μl of a cytochrome *P*-450 solution (stock solution 0.25 or 0.025 mg/ml; i.e. 0.25 or 0.025 μg of protein) on to nitrocellulose paper. The paper was then 'blocked' with a 2% (w/v) solution of bovine serum albumin dissolved in 50 mM-Tris/HCl, pH 7.9, containing 0.9% NaCl and 0.05% Tween 20 (TBST) and then exposed to the required antiserum (diluted 1:500 in TBST). The filters were then washed four times for 15 min with TBST and then exposed for 1 h to ^{125}I-labelled protein A in TBST (5 μCi/50 ml of TBST). The filters were repeatedly washed with TBST containing 0.025% SDS until no more radioactivity was eluted, and then dried before exposure to X-ray film. Western blots were carried out as described previously [14].

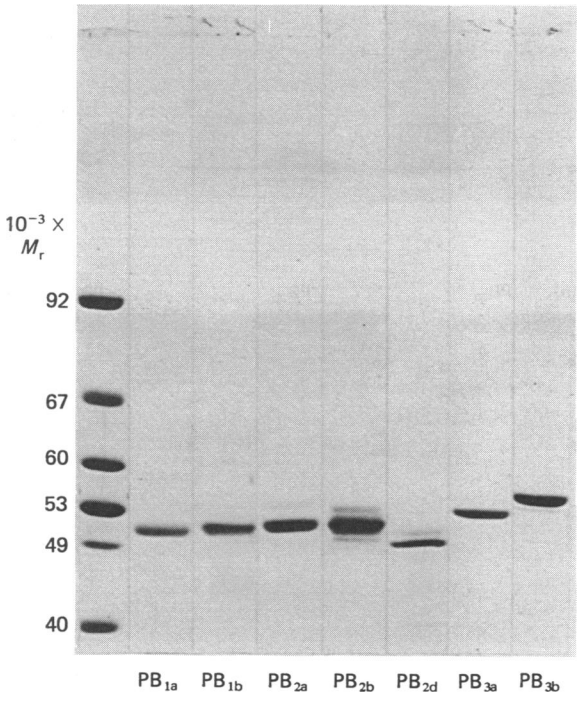


Fig. 1. SDS/polyacrylamide-gel electrophoresis of the cytochrome *P*-450 forms

The acrylamide concentration was 7.5%. A 1 μg portion of protein was run per track. Proteins were stained with Coomassie Blue. The standards used were: phosphorylase *a*, M_r 92000; bovine serum albumin, 67000; catalase, 60000; glutamate dehydrogenase, 53000; fumarase, 49000; and aldolase, 40000.

Materials

Resorufin analogues were synthesized as described elsewhere [24]. Emulgen 911 was generously given by the Kao Atlas Corporation, Tokyo, Japan. All other reagents used were from the usual commercial sources.

RESULTS

Seven cytochrome *P*-450 forms were isolated from liver microsomes of phenobarbital-treated Wistar rats (Fig. 2 below). The yields of these proteins were approx. 4, 2, 1, 0.2, 0.5, 6 and 4% of the initial cytochrome content for PB_{1a}, PB_{1b}, PB_{2a}, PB_{2b}, PB_{2d}, PB_{3a} and PB_{3b} respectively. The calculated M_r values and *N*-terminal sequences of these proteins are shown in Table 1. Only one *N*-terminal sequence was obtained for each sample,

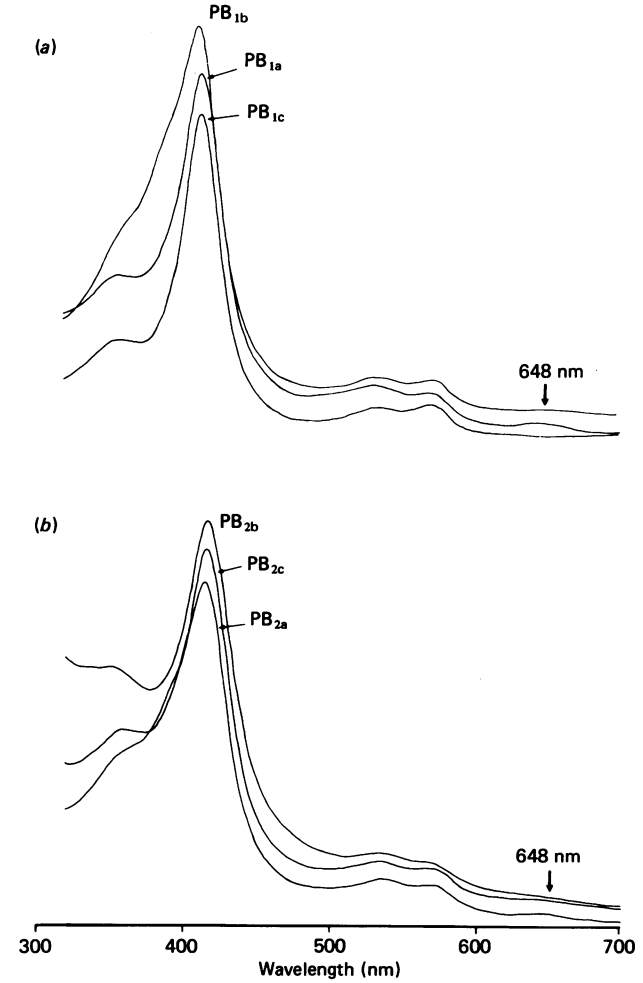


Fig. 2. Absorption spectra of various cytochrome *P*-450 isoenzymes

(*a*) Spectra of the PB_1 group of proteins; (b) spectra of the PB_2 group. Peaks observed at 648 nm are a characteristic of haem iron in the high-spin configuration.

Table 1. *N*-Terminal sequences of purified cytochromes *P*-450

Experimental details are given in the Materials and methods section. Analysis of PB_{1a} gave other possible residues in positions 1, 4 and 8; these are given in parentheses.

Cyto-chrome	Apparent M_r	Residue no. ...	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
		Sequence																				
PB_{1a}	52700		Met (Val)	Asp	Leu	Val (Met)	Met	Leu	Leu	Val (Met)	Leu	Thr	Leu	Thr	–	Leu	Ile	Leu	Leu	Ser	Ile	–
PB_{1b}	52900		Met	Asp	Leu	Val	Met	Leu	Leu	Val	Leu	Thr	Leu	Thr	–	Leu	Ile	Leu	Leu	Ser	Ile	–
PB_{2a}	52900		Met	Asp	Pro	Val	Leu	Val	Leu	Val	Leu	Thr	Leu	Ser	Ser	Leu	Leu	Leu	Leu	–	Leu	–
PB_{2a}	52900		Met	Asp	Pro	Val	Leu	Val	Leu	Val	Leu	Thr	Leu	Ser	Ser	Leu	Leu	Leu	Leu	–	Leu	–
PB_{2d}	50800		–	Asp	Leu	Val	Leu	Val	Leu	Val	Leu	Thr	Leu	–	–	Leu	Leu	Leu	Leu	Leu	–	–
PB_{3a}	53300		Met	Glu	Pro	Ser	Ile	Leu	Leu	Leu	Leu	Ala	Leu	Leu	Val	Gly	Phe	Leu	Leu	Leu	Leu	Val
PB_{3b}	54700		Met	Glu	Pro	Ser	Ile	Leu	Leu	Leu	Leu	Ala	Leu	Leu	–	Gly	Phe	Leu	Leu	Leu	Leu	Val

indicating that the proteins were of high purity. PB_{1a} and PB_{1b} had identical *N*-terminal sequences. The M_r values for PB_{1a} and PB_{1b} were very similar; however, there was a reproducible difference in their mobilities in SDS/polyacrylamide gels (Fig. 1). The M_r of PB_{1c} was 52900. PB_{2a} and PB_{2b} and PB_{2c} could not be distinguished on the basis of their mobilities in SDS/polyacrylamide gels. However, the *N*-terminal sequences of PB_{2a} and PB_{2b} were considerably different, with four out of 16 determined residues in common. PB_{2a} had 11 out of 18 residues in common with the PB_1 proteins. PB_{2d} had a very similar *N*-terminal sequence to that of PB_{2a}, the only difference being a leucine, instead of a proline, residue at position 3. However, the M_r of this protein was considerably lower than that of PB_{2a} (Fig. 1). The *N*-terminal sequences of PB_{3a} and PB_{3b} were identical; however, this sequence was significantly different from that of the other proteins.

The absolute absorption spectra of ferric cytochrome PB_{1a}, PB_{1b} and PB_{1c} are shown in Fig. 2(*a*). PB_{1b} contained a much higher proportion of high-spin haem iron characterized by the shoulder at 395 nm and the peak at 648 nm. Similar differences were observed for the

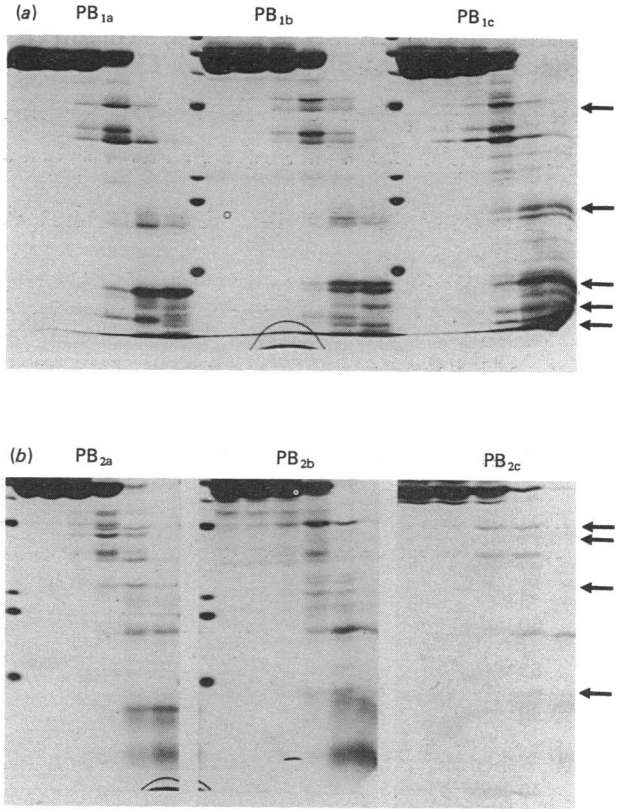


Fig. 3. Peptides produced after proteolytic (papain) digestion of cytochrome PB_1 and PB_2 proteins

Cytochrome samples [75 μg in 150 μl of 0.125 M-Tris/HCl (pH 6.8)/10% glycerol/0.05% SDS and 0.001% Bromophenol Blue] were digested with papain (40 μg) and 30 μl samples were removed at 0.5, 2.0, 5.0, 20 and 60 min. These samples were boiled with SDS/mercaptoethanol and run on SDS/12.5% (w/v)-polyacrylamide gels and then stained with Coomassie Blue. Arrows indicate the positions of differences between the proteins.

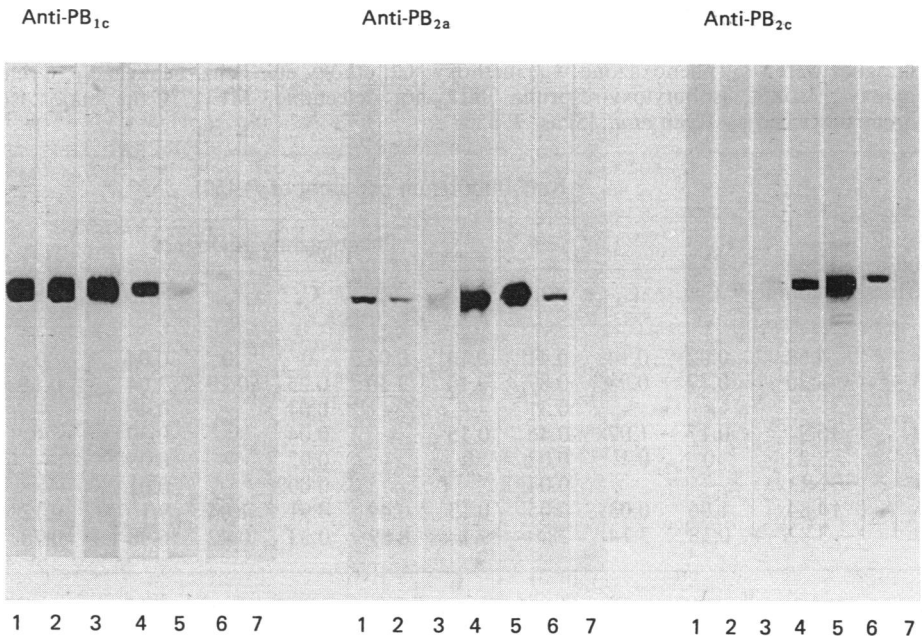


Fig. 4. Reactivity of antibodies of PB_{1c}, PB_{2a} and PB_{2c} with various cytochrome *P*-450 isoenzymes

The proteins (1 μg) were a run on SDS/9%-polyacrylamide gels and transferred to nitrocellulose and developed by using the 'Western blot' procedure as described by Adams *et al.* [14]. The rabbit antisera were used at a dilution of 1:1000 and the bands revealed by using peroxidase-labelled anti-(rabbit IgG) and 4-chloro-1-naphthol as peroxidase substrate. The bands were: 1, PB_{1a}; 2, PB_{1b}; 3, PB_{1c}; 4, PB_{2a}; 5, PB_{2b}; 6, PB_{3a}; 7, PB_{3b}. The M_r values for the proteins identified were those expected on the basis of the mobilities of the purified antigens (see Fig. 1).

Table 2. Reactivity of cytochrome *P*-450 antibodies with cytochrome *P*-450 proteins

Reactivities of the antibodies with the various cytochrome *P*-450 antigens was determined either by Ouchterlony double-immunodiffusion analysis or from dot blots. The methods used are described in the Materials and methods section. +, Very weak reactivity; ++++, very strong reactivity; –, no detectable reactivity.

		Reactivity							
Enzyme	Antibody . . .	PB_{1a}	PB_{1b}	PB_{1c}	PB_{2a}	PB_{2b}	PB_{2c}	PB_{2d}	PB_{3a}
PB_{1a}		++++	+++	+++	++	+(+)	–	+	(+)
PB_{1b}		+++	++++	+++	+	++	–	+	(+)
PB_{1c}		+++	+(+)	++++	+	++	–	+	(+)
PB_{2a}		++	++	++	++++	+(+)	++	+++	(+)
PB_{2b}		(+)	(+)	(+)	+++	++++	+++	(+)	(+)
PB_{2c}		(+)	(+)	(+)	+++	+++	++++	(+)	(+)
PB_{2d}		++	++	(+)	++	(+)	(+)	++++	(+)
PB_{3a}		(+)	(+)	(+)	+	+(+)	+	+	++++
PB_{3b}		(+)	(+)	(+)	–	–	–	(+)	++++

PB_2 proteins, where a higher proportion of PB_{2a} was in the high-spin state (Fig. 2*b*). PB_{2d} had no visible high-spin component (results not shown). These differences were not apparently due to the method of purification, as the final steps in the preparation of all these proteins was the same. Peptide maps provided further evidence which differentiated between the PB_1 proteins. The peptides produced on digestion of the PB_1 group of proteins with papain are shown in Fig. 3(*a*). Many of the peptides appeared to be the same for all three proteins; however, some differences were observed, particularly in the smaller peptide fragments. The largest differences were between PB_{1b} and the other two proteins. Peptide maps for the PB_2 group of proteins are shown in Fig. 3(*b*). Some differences between PB_{2a} and the other two cytochromes *P*-450 are apparent, but there were no clear differences beteen PB_{2b} and PB_{2c}. The peptide maps for the PB_1 forms were completely different from those obtained for the PB_2 group.

Polyclonal antibodies raised against the PB_1 proteins were used to identify structural similarities between these forms using either Western blots, dot blots or Ouchterlony immunodiffusion analysis (Fig. 4 and Table 2). Western blots obtained by using an antibody to PB_{1c},

Table 3. Metabolism of various substrates by cytochrome *P*-450 isoenzymes

The resorufin analogues were: C_0, phenoxazone; C_1, methoxy; C_2, ethoxy etc. Benz, benzyloxy-; Cyclohex, cyclohexyloxy-; Isoprop, isopropyloxy-; Isobut, isobutyloxy-resorufin. ND, not detectable; MC_{1b} is the major isoenzyme induced by 3-methylcholanthrene described by Ryan *et al.* [5] as '*P*-450c'.

	Rate (nmol/min per nmol of *P*-450)												
			Phenoxazone analogues										
Enzyme	7-Ethoxy-coumarin	Benz-phetamine	C_0	C_1	C_2	C_3	C_4	C_5	C_6	Benz	Cyclohex	Isoprop	Isobut
PB_{1a}	0.31	4.62	0.02	0.14	0.41	0.14	0.04	0	0	0.04	0	0.15	0.01
PB_{1b}	0.33	3.20	0.17	0.98	0.87	2.14	2.30	0.25	0.19	3.04	0.15	0.26	0.10
PB_{1c}	3.4	–	–	–	0.31	–	–	0.04	–	0.40	–	–	–
PB_{2a}	0.69	15.9	0.17	1.02	0.46	0.15	–	0.04	–	0.30	–	–	–
PB_{2b}	0.17	3.2	0	0.1	0.02	0	–	0.02	–	0.08	–	–	–
PB_{2c}	ND	ND	–	–	0.04	–	–	0.002	–	0.01	–	–	–
PB_{3a}	2.06	14.84	1.06	0.03	0.05	0.13	0.49	4.91	1.04	9.1	0.12	0.08	0.09
MC_{1b}	56.69	3.82	0.18	3.14	28.0	39.1	8.89	0.33	0.42	6.98	0.78	18.32	2.07

PB_{2a} and PB_{2c} are shown in Fig. 4. Each of the antibodies raised against the PB_1 group reacted with all the members of the PB_1 group and, to a lesser extent, with PB_{2a} and PB_{2d}. These antibodies did not react significantly with PB_{2b} and PB_{2c} or PB_{3a} and PB_{3b} (Table 2). The antibody to PB_{2a} reacted strongly with PB_{2b} and PB_{2c} and weakly with PB_1 and PB_{3a} (Fig. 4; Table 2) and, interestingly, not with PB_{3b}. The antibody to PB_{2b} reacted quite strongly with all the PB_1/PB_2 proteins with the exception of PB_{2d}. The PB_{2c} antibody reacted strongly with PB_{2a} and PB_{2b}, but did not react with any of the PB_1 group. The reactivity of the PB_{2d} antibody was similar to that of PB_{2a}, but with lower reactivity towards PB_{2b} and PB_{2c}.

The substrate specificities of the PB_1 and PB_2 proteins are shown in Table 3. Significant differences in activity were observed among the PB_1 group. PB_{1a} and PB_{1b} had similar benzphetamine *N*-demethylation activities, although PB_{1a} was less active than PB_{1c} in the metabolism of 7-ethoxycoumarin. However, PB_{1b} had in general the highest activities towards the phenoxazone homologues. A major difference was in the metabolism of benzyloxyresorufin, the activity of PB_{1b} being 76-fold and 7-fold higher than PB_{1a} and PB_{1c} respectively. This substrate therefore also distinguished between PB_{1a} and PB_{1c}. Large differences between PB_{1a} and PB_{1b} were also observed in the metabolism of the methoxy and propyloxy derivatives.

In the case of the PB_2 proteins, PB_{2a} had much higher activity than PB_{2b} or PB_{2c} towards all the substrates tested. The exceptionally high activity of PB_{2a} towards benzphetamine was of interest, being comparable with the activity of PB_{3a}. The preferred phenoxazone derivative for PB_{2a} was the methoxy homologue, in contrast with the benzyloxy homologue for PB_{1b} and PB_{3a}. The results for all of the different forms of cytochrome *P*-450 encompass substrate selectivities for each of the C_0-C_5 substrates; i.e. for C_0 (phenoxazone) and C_5 (pentoxyphenoxazone), PB_{3a}; for C_1, PB_{2a}; for C_2 and C_3, MC_2; and for C_3 and C_4, PB_{1b}.

DISCUSSION

In a previous report [12] we have described the isolation of two distinguishable forms of rat liver cytochrome *P*-450 (PB_1 and PB_2), which are only marginally induced by phenobarbital relative to certain other forms, namely PB_{3a} and PB_{3b} [5,6,8]. We have now isolated a series of proteins that appear structurally related to PB_1 and PB_2. The use of the homologous series of phenoxazone substrates proved to be a powerful method for distinguishing between many different forms of cytochrome *P*-450 [25] is clearly demonstrated here. The degree of specificity of forms PB_{1b}, PB_{2a}, PB_{3a} and MC_2 is remarkable, with changes in substrate structure of just one methylene unit in the side chain. On this basis and also antibody reactivity and *N*-terminal-sequence analysis three members of the PB_1 group of proteins have been tentatively identified. Although they appear to have identical *N*-terminal sequences, PB_{1a} and PB_{1b} can be clearly distinguished from each other by their different M_r values, spectra and substrate specificities. Although PB_{1c} appears to be another member of this group, it was isolated from a different microsomal preparation by a different purification procedure [12]. The *N*-terminal sequence of PB_{1a} and PB_{1b} are identical with that of the enzyme described by Waxman & Walsh [11] as PB_1. These data demonstrate that several gene products of this *P*-450 subfamily are expressed with identical *N*-terminal sequences. The finding that PB_{1b} contained a significant proportion of high-spin haem iron was interesting, particularly in view of the finding that PB_{2a}, PB_{3b} (not shown) and MC_1 [5,21] all have a significant high-spin component. The reason and implications of this observation in relation to cytochrome *P*-450 function are obscure. Within the group of proteins named PB_2, PB_{2a} and PB_{2b} can be distinguished from each other by their substrate specificity, immunochemical properties and *N*-terminal sequences. The *N*-terminal sequence of PB_{2a} was identical with that described by Hanniu *et al.* [26] as 'form h' and by Waxman [27] as 'form 2c', by Cheng & Schenkman as 'RLM_5' [28] and by Kamataki *et al.* [29] as '*P*-450 male'. Form 2d appears to be a protein different from, but structurally related to, PB_{2a}. It had a different M_r, did not metabolize benzphetamine (result not shown) and had one difference in the *N*-terminal sequence. The first three *N*-terminal amino acids of PB_{2d} are the same as 'form f' described by Hanniu *et al.* [26]; however, the sequence is then identical with form h and not form f. Those authors have discussed whether form

h was equivalent to that described by Waxman & Walsh [11] as PB_1. From these studies, and in substantiation of their hypothesis, it is clear that these represent different proteins which are both expressed simultaneously. In the absence of full-length amino acid sequences and chromosomal localizations it is difficult to assess which cytochrome forms belong within the same gene subfamily. The similarity in the *N*-terminal sequences of PB_{2a} and PB_{2d} with the PB_1 proteins indicates that they may belong to the same subgroup (however, the peptide maps and antibody reactivity indicate homology with PB_{2b} and PB_{2c}). Ryan *et al.* [30] have described four closely related enzymes termed f, g, h and i (form i being a female-specific enzyme) which may also be in this subgroup. Form h is equivalent to PB_{2a}. Therefore, forms f, g, and i together with PB_{1a}, PB_{1b}, PB_{2a}, PB_{2b} and PB_{2d} would indicate that at least seven members of this subfamily are expressed. The *N*-terminal sequence of PB_{2b} had 13 out of 16 residues identical with two cDNAs belonging to pregnenolone-16-α-carbonitrile-inducible *P*-450 species (Gonzales *et al.* [31]) and would appear to be another member of this subfamily of proteins and therefore distinct from the PB_1 group. However, more work is required to unequivocally place these proteins together. It is interesting that Gonzales *et al.* [31] reported that an mRNA species derived from a gene within this subgroup was only inducible with phenobarbital and not with pregnenolone-16-α-carbonitrile. The structural similarity between PB_{2b} and PB_{2c} and the PB_1 proteins also requires further study. The chromosomal localization of the genes coding for these proteins will prove important in their classification. PB_{2b} was structurally very similar to PB_{2c} and, as it was obtained from a different source, may prove to be the same protein. The structural similarity of PB_{2b} and PB_{2c} with the other proteins would appear to be related to regions not related to the *N*-terminal of the protein. PB_{2b} (PB_{2c}) on the basis of yield from the purification, appears to be present in rat microsomal fractions in low concentration. From previous studies PB_{2c} was shown to be phenobarbital-inducible and highly localized in the centrilobular region of the liver [12], in contrast with the PB_1 proteins, which are more diffusely distributed in this area [12]. We have also shown that both PB_1- and PB_2-related proteins are expressed in significant concentrations in human liver, but differences in the level of expression are observed between individuals. It is interesting that antibodies to both PB_{1c} and PB_{2c} recognized two proteins in human liver. We have recently isolated cDNA probes for the human PB_1 subfamily (R. Meehan, N. Hastie, T. Friedberg, M. Adesnik & C. R. Wolf, unpublished work), and Southern-blot analysis indicates that the PB_1 gene subfamily comprises several genes in man.

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REFERENCES

1. Nebert, D. W. & Gonzalez, F. J. (1985) Trends Pharmacol. Sci. **165**, 160–164
2. Lu, A. Y. H. & West, S. B. (1980) Pharmacol Rev. **31**, 277–295
3. Adesnik, M. & Atchison, M. (1986) CRC Crit. Rev. Biochem. **19**, 247–305
4. Wolf, C. R. (1986) Trends Genet. **2**, 209–214
5. Ryan, D. E., Thomas, P. E., Reik, L. M. & Levin, W. (1982) Xenobiotica **12**, 727–744
6. Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, V. & Kaminsky, L. S. (1982) Biochemistry **21**, 6019–6030
7. Heuman, D. M., Gallagher, E. J., Barwick, J. L., Elshourbagy, N. A. & Guezelian, P. S. (1982) Mol. Pharmacol. **21**, 753–760
8. Kumar, A., Raphael, C. & Adesnik, M. (1983) J. Biol. Chem. **258**, 11280–11284
9. Suwa, Y., Mizukami, Y., Sogawa, K. & Fujii-Kuriyama, Y. (1984) J. Biol. Chem. **260**, 7980–7984
10. Ryan, D. E., Thomas, P. E. & Levin, W. (1982) Arch. Biochem. Biophys. **216**, 272–288
11. Waxman, D. J. & Walsh, C. (1983) Biochemistry **22**, 4846–4855
12. Wolf, C. R., Moll, E., Friedberg, T., Oesch, F., Buchmann, A., Kuhlmann, W. D. & Kunz, W. (1984) Carcinogenesis **5**, 993–1001
13. Buchmann, A., Kuhlmann, W., Schwarz, M., Kunz, W., Wolf, C. R., Moll, E., Friedberg, T. & Oesch, F. (1985) Carcinogenesis **6**, 513–521
14. Adams, D. J., Seilman, S., Amelizad, Z., Oesch, F. & Wolf, C. R. (1985) Biochem. J. **232**, 869–876
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
16. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
17. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) J. Biol. Chem. **252**, 1102–1106
18. Slaughter, S. R., Wolf, C. R., Marciniszyn, J. P. & Philpot, R. M. (1981) J. Biol. Chem. **256**, 2499–2503
19. Wolf, C. R., Smith, B. R., Ball, L. M., Serabjit-Singh, C. J., Bend, J. R. & Philpot, R. M. (1979) J. Biol. Chem. **254**, 3658–3663
20. Nash, T. (1953) Biochem. J. **55**, 416–421
21. Wolf, C. R. & Oesch, F. (1983) Biochem. Biophys. Res. Commun. **111**, 504–511
22. Ullrich, V. & Weber, P. (1972) Hoppe-Seyler's Z. Physiol. Chem. **353**, 1171–1177
23. Burke, M. D. & Mayer, R. T. (1974) Drug Metab. Dispos. **2**, 583–588
24. Burke, M. D. & Mayer, R. T. (1983) Chem.-Biol. Interact. **45**, 243–256
25. Burke, M. D., Thompson, S., Elcombe, G. R., Halpert, J., Haaparanta, T. & Mayer, R. T. (1985) Biochem. Pharmacol. **34**, 3337–3345
26. Hanniu, M., Ryan, D. E., Iida, S., Lieber, C. S., Levin, W. & Shivley, J. E. (1984) Arch. Biochem. Biophys. **235**, 304–311
27. Waxman, D. J. (1984) J. Biol. Chem. **259**, 15481–15490
28. Cheng, K. C. & Schenkman, J. B. (1982) J. Biol. Chem. **257**, 2378–2385
29. Kamataki, T., Maeda, K., Yamazoe, Y., Nagai, T. & Kato, R. (1983) Arch. Biochem. Biophys. **225**, 758–770
30. Ryan, D. E., Iida, S., Wood, A. W., Thomas, P. E., Lieber, C. S. & Levin, W. (1984) J. Biol. Chem. **259**, 1239–1250
31. Gonzales, F. J., Song, B. Y. & Hardwick, J. P. (1986) Mol. Cell Biol. **6**, 2969–2976